RNase E/G-dependent degradation of metE mRNA, encoding methionine synthase, in Corynebacterium glutamicum.

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Running title: Degradation of metE mRNA by RNase E/G

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Summary

*Corynebacterium glutamicum* is used for the industrial production of various metabolites, including L-glutamic acid and L-lysine. With the aim of understanding the post-transcriptional regulation of amino acid biosynthesis in this bacterium, we investigated the role of RNase E/G in the degradation of mRNAs encoding metabolic enzymes. In this study, we found that the cobalamin-independent methionine synthase MetE was overexpressed in ΔrneG mutant cells grown on various carbon sources. The level of *metE* mRNA was also approximately 6- to 10-fold higher in the ΔrneG mutant strain than in the wild-type strain. A rifampicin chase experiment showed that the half-life of *metE* mRNA was approximately 4.2 times longer in the ΔrneG mutant than in the wild-type strain. These results showed that RNase E/G is involved in the degradation of *metE* mRNA in *C. glutamicum*.

Keywords: *Corynebacterium glutamicum*; *metE*; mRNA degradation; RNase E/G

Introduction

*Corynebacterium glutamicum* is a Gram-positive, nonpathogenic soil bacterium that is a workhorse for amino acid production. More than 2.5 million tons of the flavor enhancer L-glutamic acid and one million tons of the feed additive L-lysine per year are produced using *C. glutamicum* (Hirasawa and Shimizu, 2016). This bacterium has also been widely used for the
production of various other metabolites, including D-amino acids, organic acids, diamines, fuels, proteins, and aromatic compounds (Becker and Wittmann, 2012; Matsuda et al., 2014; Kogure et al., 2016; Kubota et al., 2016). *C. glutamicum* belongs to the mycolic acid-containing actinomycetes, which also includes the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus*.

Ribonucleases (RNases) play an important role in the post-transcriptional regulation of gene expression (Mackie, 2013). In most bacteria, such as *E. coli*, mRNA decay depends on the initial cleavage, which is mainly catalyzed by RNase E/G family enzymes (Arraiano et al., 2010). RNase E/G endoribonucleolytically cleaves single-stranded AU-rich regions (Mackie, 2013). In *E. coli*, mRNA decay is often initiated by RNase E via one of two pathways. In one pathway, RNase E directly accesses internal sites in target mRNAs with 5′-terminal triphosphate (Clarke et al., 2014). The other pathway is 5′ end-dependent, and it is stimulated by the removal of two of the three phosphates from the 5′ terminus of primary transcripts (Celesnik et al., 2007; Deana et al., 2008; Luciano et al., 2017). *E. coli* has two RNase E/G family enzymes, RNase E and RNase G, and RNase E is essential for cell viability in *E. coli* (Apprion and Lassar, 1978; Arraiano et al., 2010). In contrast, *C. glutamicum* has only one RNase E/G encoded by *rneG*, and it is not essential (Maeda and Wachi, 2012a). The *C. glutamicum* RNase E/G is involved in 5′-end processing of the 5S rRNA and 3′-end processing of the 4.5S RNA (Maeda and Wachi, 2012a; Maeda et al., 2017). In addition, the RNase E/G degrades *aceA* mRNA, which encodes the glyoxylate cycle enzyme isocitrate lyase. Degradation of *aceA* mRNA by RNase E/G depends on the 3′-untranslated region (3′-UTR) (Maeda and Wachi, 2012b). It was also reported that the transcriptional termination factor Rho and RNase E/G play a central role in FMN riboswitch regulation in *C. glutamicum* (Takemoto
In C. glutamicum, both Rho and RNase E/G are individually dispensable, whereas simultaneous disruption is synthetic lethal (Takemoto et al., 2015).

In this study, we showed that RNase E/G is involved in the degradation of metE mRNA encoding cobalamin-independent methionine synthase in C. glutamicum.

Materials and Methods

Mediad and growth conditions. L broth containing 1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.0) was used as the complex medium. CGC medium (Eikmanns et al., 1991) containing an appropriate carbon source, 5.0 g/L (NH₄)₂SO₄, 5.0 g/L urea, 21 g/L morpholinepropanesulfonic acid (MOPS), 1.0 g/L K₂HPO₄, 1.0 g/L KH₂PO₄, 0.25 g/L MgSO₄·7H₂O, 0.01 g/L CaCl₂, 16.4 mg/L FeSO₄·7H₂O, 10 mg/L MnSO₄·H₂O, 0.2 mg/L CuSO₄·5H₂O, 1.0 mg/L ZnSO₄·7H₂O, 0.2 mg/L NiCl₂·6H₂O, 0.2 mg/L biotin, and 1.0 mg/L thiamine (pH 6.8) was used as the minimal medium. When necessary, media were supplemented with 20 μg/mL kanamycin. Glucose, fructose, sucrose, ribose, L-arabinose, sodium acetate, sodium lactate, or sodium gluconate (1% w/v) was added as a carbon source. C. glutamicum was grown aerobically at 30°C, and cell growth in liquid medium was monitored by measuring the optical density at 660 nm (OD₆₆₀). E. coli JM109 [recA1 endA1 gyrA96 thi hsdR17 supE44 relA Δ(lac-proAB)/F’(traD36 proAB¹ lacF¹ lacZΔM15)] and JM110 [dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx Δ(lac-proAB)/F’(traD36 proAB¹ lacF¹ lacZΔM15)] cells were used for all genetic manipulations. E. coli strains were cultivated at 37°C in L medium supplemented with 50 μg/mL kanamycin when necessary.
**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. A 3.14-kb DNA fragment containing the *metE* gene with 445 bp upstream region, which contains its own promoter sequence, and 455 bp downstream region, which contains a Rho-dependent terminator-like sequence, was amplified by PCR using the primers 5′-CGCGTTGAATTCTGCAAAACCCC-3’ and 5′-GGGAATGTCGACGCAGTTGGCCC-3’ (the artificially generated EcoRI and SalI sites are underlined). The amplified fragment was digested with EcoRI and SalI, and then the digested DNA fragment was cloned into the *E. coli-C. glutamicum* shuttle vector plasmid pEctS (Maeda and Wachi, 2012b) digested with the same enzymes to construct plasmid pCmE-FL.

**Analysis of cellular proteins.** The total cellular proteins of *C. glutamicum* were prepared as described previously (Maeda and Wachi, 2012b). Briefly, cells were suspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. After removal of the unbroken cells, the proteins in the lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel was stained with Coomassie brilliant blue. The 80-kDa protein band was cut out and analyzed by mass spectrometry (MALDI-TOF/TOF ultrafleXtreme; Bruker Daltonics, Inc., Billerica, MA) after in-gel trypsin digestion.

**Total RNA purification.** Total cellular RNA from *C. glutamicum* cells was isolated as described previously (Maeda and Wachi, 2012b). Briefly, overnight cultures grown in CGC
medium containing a carbon source (1%) at 30°C were washed and then inoculated into fresh
CGC minimal medium containing a carbon source (1%). Then, two volumes of RNA Protect
bacterial reagent (Qiagen, Valencia, CA) were added directly to one volume of exponentially
growing cultures (OD$_{660}$ of ~1; mid-exponential growth phase) to stabilize cellular RNA. The
cells were harvested by centrifugation at 5,000×g for 10 min at 25°C, and total cellular RNA
was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions.
Total RNA was treated with DNase I at 37°C for 1 h.

**qRT-PCR.** The mRNA was quantified using the Eco Real-Time PCR System (Illumina,
Inc., San Diego, CA) as described previously (Maeda and Wachi, 2012b). Primers used in this
quantitative real-time PCR (qRT-PCR) analysis are listed in Table 2. Briefly, 50 ng of total
RNA was used in each RT-PCR using the QuantiFast SYBR green RT-PCR kit (Qiagen)
according to the manufacturer’s instructions. Negative controls, with no reverse transcriptase,
were included for each RNA sample to rule out genomic DNA contamination. Target gene
transcript levels were normalized to the reference gene transcript (16S rRNA) in the same RNA
sample. Each gene was analyzed using RNA isolated from at least three independent samples.

**Results**

**Overproduction of MetE protein in the ΔrneG strain**

We previously reported that isocitrate lyase encoded by *aceA* was overproduced in
*ΔrneG* mutant cells grown on acetate as a single carbon source (Maeda and Wachi, 2012b). We
performed a proteome analysis using cells grown to a stationary phase on various sugars and organic acids as a sole carbon source (Maeda and Wachi, 2012b). In the present study, we compared the protein expression patterns between wild-type and ΔrneG cells in the exponential growth phase to identify additional target mRNAs of RNase E/G. Total cellular proteins obtained from cells grown to the mid-exponential growth phase in complex or minimal medium with glucose, fructose, sucrose, ribose, L-arabinose, acetate, lactate, or gluconate as the sole carbon source were separated by SDS-PAGE. As shown in Fig. 1, a protein of ~80-kDa was overproduced in ΔrneG cells grown on fructose or sucrose as the sole carbon source, and as previously reported, AceA was overproduced in ΔrneG cells grown on acetate (Maeda and Wachi, 2012b). Overproduction of the 80-kDa protein was not observed apparently when the cells were grown on other carbon sources. This 80-kDa protein band was identified as MetE by mass spectrometry, with a deduced molecular mass of 81.3 kDa. The number of peptides matched and sequence coverage were 29 and 64.3% respectively for MetE in MASCOT analysis (Matric Science Ltd., London, UK). To observe the effect of the ΔrneG mutation on the expression of metE more precisely, we constructed a metE-overexpressing plasmid named pCmE-FL, which harbors the metE gene with its own promoter and terminator. Then, wild-type and ΔrneG mutant strains harboring pCmE-FL were grown on glucose, fructose, or acetate as the sole carbon source and the cells were harvested at the mid-exponential growth phase. As shown in Fig. 2, MetE overexpression in ΔrneG mutant cells harboring pCmE-FL was not clearly affected by the carbon source. This result indicates that overproduction of MetE in exponentially growing ΔrneG cells is independent of the carbon source. It is possible that the basal expression levels of metE were higher when cells were grown on fructose or sucrose than
those on other carbon sources, which made the overproduction of MetE protein more prominent in the ΔrneG mutant grown on fructose or sucrose in Fig. 1.

We also examined whether ΔrneG cells overproduced MetE protein at the stationary phase. Wild-type and ΔrneG mutant strains harboring pCmE-FL were grown on fructose as a sole carbon source, and cells were harvested at both the mid-exponential growth phase and stationary phase. As shown in Fig. 3, the ΔrneG mutant harboring pCmE-FL overproduced MetE at the exponential growth phase, but this was not so obvious at the stationary phase. Even in the wild-type cells, the expression level of MetE protein was decreased at the stationary phase compared with that at the exponentially growing phase.

**Increased stability of metE mRNA in ΔrneG cells**

The proteome analysis shown in Figs. 1–3 suggested that overproduction of MetE in exponentially growing ΔrneG cells resulted from a defect in metE mRNA degradation by RNase E/G. To examine the effect of the ΔrneG mutation on the stability of metE mRNA, we first performed a qRT-PCR analysis. Total RNA was extracted from wild-type and ΔrneG cells growing exponentially on glucose, fructose, or acetate as a sole carbon source. As shown in Fig. 4, the level of metE mRNA in ΔrneG cells was approximately 6.1-fold higher than that in wild-type cells when grown on fructose as the sole carbon source. In addition, increased levels of metE mRNA were also observed in the ΔrneG mutant when the cells were grown on glucose or acetate as the sole carbon source. The levels of metE mRNA in the ΔrneG mutant were approximately 10.8-fold and 9.7-fold higher than that in the wild-type cells when grown on either glucose or acetate as the sole carbon source, respectively (Fig. 4). These results suggest
that RNase E/G degrades metE mRNA regardless of the carbon source.

We also examined mRNA levels at the stationary phase. The expression levels of metE mRNA were decreased to about 1/20 both in wild-type and ΔrneG mutant cells at the stationary phase compared with those at the exponential phase; that is, the increased levels of metE mRNA in ΔrneG mutant were still observed in the stationary phase (data not shown). This suggests that RNase E/G degrades metE mRNA both at the exponential and stationary phase. It is possible that the effect of the ΔrneG mutation is more prominent for highly expressed genes when observed at protein levels. Previously identified RNase E/G target mRNAs, adhE of E. coli and aceA of C. glutamicum, are also highly expressed genes (Umitsuki et al., 2001; Maeda and Wachi, 2012b).

Next, we measured the half-lives of metE mRNA in wild-type and ΔrneG cells grown on fructose as a sole carbon source. Exponentially growing cells were treated with 150 µg/mL rifampicin to prevent further initiation of transcription. Then, total RNA was isolated at various time points after the addition of rifampicin, and the rates of decay of metE mRNA were determined in wild-type and ΔrneG cells (Fig. 5). metE mRNA showed a half-life of 2.1 min in wild-type cells, and a prolonged half-life of 8.9 min in ΔrneG cells. These results indicated that the overexpression of MetE in the ΔrneG mutant results from the increased stability of metE mRNA.

Discussion

In this study, we have, for the first time, identified metE mRNA as a substrate of
RNase E/G, in addition to aceA mRNA. We showed that MetE was overproduced in ΔrneG mutant cells when compared to the levels in wild-type cells during the exponential growth phase. The levels of metE mRNA were also increased in ΔrneG mutant cells. A rifampicin chase experiment showed that the half-life of metE mRNA was prolonged in ΔrneG mutant cells. These results indicate that RNase E/G is involved in the degradation of metE mRNA in C. glutamicum.

Methionine is synthesized from homoserine in C. glutamicum. Homoserine is converted to O-acetylhomoserine by MetX (homoserine O-acetyltransferase) (Rückert et al., 2003). Then, homocysteine is synthesized by a trans-sulfuration pathway with MetB (cystathionine γ-synthase) and MetC (cystathionine β-lyase), or direct sulphydrylation with MetY (O-acetylhomoserine sulphhydrylase) (Lee and Hwang, 2003; Rückert et al., 2003). The final step in L-methionine biosynthesis is catalyzed by methionine synthase. C. glutamicum possesses two methionine synthases, one encoded by metE and the other encoded by metH (Rückert et al., 2003). MetE is cobalamin (vitamin B₁₂) independent, whereas MetH is cobalamin dependent. Our preliminary experiment showed that a ΔmetE mutant was auxotrophic for methionine, suggesting that MetE but not MetH plays a major role in methionine synthesis, at least, under our experimental conditions (data not shown). The transcriptional repressor McbR regulates the expression of almost all enzymes involved in methionine biosynthesis (Rey et al., 2003; Rey et al., 2005; Suda et al., 2008). Methionine production by fermentation has been explored by overproducing biosynthetic enzymes and/or deleting McbR in C. glutamicum, but productivity was still low for commercial purposes (Park et al., 2007; Qin et al., 2015; Li et al., 2016). Overproduction of MetE by the rneG mutation
might be applied for improving the productivity of methionine.

It was previously shown that acidic stress induced oxidative stress in *C. glutamicum*. Oxidative stress caused MetE inactivation by S-mycothiolation, which resulted in methionine synthesis impairment. Under these conditions, MetE protein levels were increased (Follmann et al., 2009, Liu et al., 2016). In *E. coli*, it is also reported that oxidative stress causes MetE inactivation by S-glutathionylation, which results in methionine limitation (Hondorp and Matthews, 2004). Since rapid mRNA degradation permits fast change in gene expression, the degradation of *metE* mRNA by RNase E/G might be involved in the stress response mechanism.

Several studies in *E. coli* and other bacteria have shown that mRNA decay is frequently initiated by RNase E cleavage in the 5′-UTR (Kaberdin and Bläsi, 2006; Hankins et al., 2007). The 5′-UTRs can contain regulatory elements involved in up- or down-regulation of translation, including ribosome binding signals, translation repressors, low molecular-weight effectors, and small RNA with corresponding effects on mRNA stability (Kaberdin and Bläsi, 2006). In contrast, strong stem-loop structures such as the transcription terminator at the 3′ end of many bacterial mRNAs are resistant to 3′ to 5′ exoribonucleolytic degradation (McLaren et al., 1991; Spickler and Mackie, 2000). Our previous study showed that the degradation of *adhE* mRNA was 5′-UTR dependent in *E. coli* (Umitsuki et al., 2001; Ito et al., 2013). We also showed that RNase E/G cleavage of *aceA* mRNA is dependent on the 3′-UTR in *C. glutamicum* (Maeda and Wachi, 2012b). The 3′-UTR of *aceA* mRNA contains a typical Rho-independent terminator. RNase E/G cleavage removes this Rho-independent terminator and generates an unprotected 3′ end, which is rapidly degraded by 3′ to 5′ exoribonucleases (Maeda and Wachi,
In these studies, lacZ assay fused with 5′- or 3′-UTR was valid for estimating cleavage sites. Therefore, we constructed plasmids harboring metE 5′-UTR-lacZ and lacZ- metE 3′-UTR fusion genes. However, neither metE 5′-UTR-lacZ nor lacZ- metE 3′-UTR showed significant difference in the expression between wild-type and ΔrneG strains (data not shown). This suggests that the mechanism for metE mRNA degradation by RNase E/G is different from 5′- or 3′-UTR dependent mechanism. Unlike in aceA mRNA, a Rho-dependent terminator-like sequence (i.e., C-rich and G-poor sequences that lack obvious secondary structure followed by a boxA signal) was found at the 3′ end of the metE transcript (Friedman and Olson, 1983; Richardson and Richardson, 1996). It was previously shown that RNase III-mediated degradation of mraZ mRNA in C. glutamicum is coding region dependent (Maeda et al., 2016). Secondary structures within the coding region would slow down translation by ribosomes, which consequently increases the probability of degradation by endoribonucleases such as RNase III and RNase E/G (Braun et al., 1998; Maeda et al., 2016). Determination of RNase E/G cleavage site in metE mRNA remains to be done.

Acknowledgements

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References


Functional genomics of pH homeostasis in *Corynebacterium glutamicum* revealed novel links between pH response, oxidative stress, iron homeostasis and methionine synthesis.


Kubota, T., Watanabe, A., Suda, M., Kogure, T., Hiraga, K., and Inui, M. (2016) Production of para-aminobenzoate by genetically engineered *Corynebacterium glutamicum* and


Figure legends

Fig. 1. Overproduction of MetE protein in the ΔrneG mutant. ATCC31831 (wild-type [+] and D2281 (ΔrneG [-]) cells were grown to the mid-exponential growth phase in media with various sugars and organic acids as the sole carbon source. Then, the total cellular proteins were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The positions of the 80-kDa protein and AceA are shown on the right of the gel. The carbon sources are shown at the top of the gel. L: L broth, Glu: glucose, Fru: fructose, Suc: sucrose, Rib: ribose, Ara: L-arabinose, Ace: acetate, Lac: lactate, and Gln: gluconate.

Fig. 2. The effect of the carbon source on MetE overproduction in ΔrneG mutant cells. Wild-type (+) and ΔrneG (-) mutant cells harboring a metE overexpression plasmid (pCmE-FL) were grown to the mid-exponential growth phase on glucose, fructose, or acetate as the sole carbon source. The total cellular proteins were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The position of MetE is shown on the right of the gel. The carbon sources are shown at the top of the gel. A protein band (indicated by an arrowhead) migrated just below MetE, which is overproduced in the wild-type cells harboring pCmE-FL grown on acetate, is malate synthase encoded by aceB. The expression of aceB is induced when grown on acetate.

Fig. 3. The effect of the growth phase on MetE overproduction in ΔrneG mutant cells. Wild-type (+) and ΔrneG (-) mutant cells harboring the pCmE-FL plasmid were grown to the
mid-exponential growth phase or stationary phase on fructose as the sole carbon source. Then, the total cellular proteins obtained were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The position of MetE is shown on the right of the gel.

**Fig. 4.** Relative metE mRNA levels in wild-type and ΔrneG cells grown on glucose, fructose, or acetate as the sole carbon source. The total cellular RNA was extracted from exponentially growing cells. Relative metE mRNA levels were measured by qRT-PCR and normalized to 16S rRNA transcript levels. The levels are presented relative to that in wild-type cells, which was set to 1. The values are the means (with standard deviation) from at least three independent experiments.

**Fig. 5.** Increased stability of metE mRNA in ΔrneG mutant cells. The half-lives of metE mRNA in wild-type (open triangles) and ΔrneG mutant (filled circles) cells were determined. Rifampicin was added to exponentially growing cultures at time zero, and at the indicated time points, the total RNA was isolated and analyzed by qRT-PCR using specific primers for metE mRNA and normalized to 16S rRNA transcript levels. The half-lives were calculated from three independent experiments with standard deviations.
### Table 1. *C. glutamicum* strains and plasmids used in this study

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### Table 2. Primers used in qRT-PCR analysis

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Fig. 1
Fig. 2

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(kDa)

200.0, 116.0, 97.4, 66.4, 44.3

rmeG + - + - + - + - + -

MetE
Fig. 3
Fig. 4
Fig. 5